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Lack of complement activation by human IgA immune complexes

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SUMMARY

Complement activation may play an important role in renal injury associated with glomerular deposition of IgA immune complexes. The ability of naturally occurring human IgA immune complexes (IgA-IC) and covalently cross-linked human IgA oligomers (X-IgA) to activate complement were examined *in vitro* and *in vivo*. Large-sized IgA-IC were isolated from a patient's serum by affinity purification (Jacalin-Sepharose) and gel chromatography. Stable X-IgA were prepared by chemical cross-linking with a heterobifunctional reagent, *N*-succinimdyl 3-(2-pyridyl-dithio) propionate (SPDP)., Treatment of fresh normal human serum with large amounts of either IgA-IC or X-IgA failed to activate C3. The C3 consumption was measured immunochemically by the decrease of the B antigen on the native C3 and by the generation of iC3b. Addition of these complexes to serum did not result in cleavage of factor B. Administration of human IgA-IC or X-IgA to mice, killed after 6 h, resulted in glomerular deposition of IgA. Despite the presence of intense glomerular IgA deposits no C3 was detected. Collectively, these findings suggest that neither soluble nor renal localized human IgA complexes activate complement.

Keywords complement IgA immune complexes nephritis

INTRODUCTION

Glomerular IgA deposits represent the diagnostic hallmark of primary IgA nephropathy and Henoch–Schönlein nephritis (Clarkson *et al.*, 1987). An immune complex aetiology of these diseases is suggested by the similar glomerular distribution of granular pattern IgA deposits by immunofluorescent microscopy and dense deposits by electron microscopy. Further support for such a mechanism is the detection of IgA immune complexes (IgA-IC) in patients with these conditions (Levinsky & Barratt, 1979; Valentijn *et al.*, 1983). Glomerular deposits of the third complement component (C3) and properdin are often but not necessarily present in a similar pattern to the IgA. Although C3 activation fragments may be demonstrable in the circulation of many patients (Wyatt *et. al.*, 1987), the serum levels of C3 and properdin are usually normal (Julian *et al.*, 1983).

The concept that human IgA immune complexes activate the complement alternative pathway is based on reports showing that chemical or interfacial aggregates of human IgA myeloma proteins promote limited consumption in human serum of the haemolytic complement (C3-C9) activity (Gotze & Muller-Eberhard, 1971; Boackle, Pruitt & Mestecky, 1974). In contrast, the secretory IgA anti-blood group A bound to the target blood cells failed to activate complement (Colten & Bienenstock, 1976). While most of the above studies appear to implicate IgA-IC in the activation of the alternative complement pathway in patients with IgA nephropathy, direct evidence that this activation is accomplished by IgA-IC alone is lacking.

The present study was designed to investigate the interaction *in vitro* and *in vivo* of serum complement and purified human IgA immune complexes or covalently cross-linked IgA oligomers (X-IgA).

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MATERIALS AND METHODS

Purification of IgA

IgA was purified from 100 ml of pooled normal human sera by a two-step procedure. In the first step, the IgA was precipitated with 33% ammonium sulphate. This was repeated twice after resolubilization of the precipitate with distilled water. The final solution was adjusted to 0·15 M NaCl with 4·0 M NaCl. In the second step, the IgA-containing solution was applied to a 50 ml Jacalin-Sepharose 4B column for affinity purification as described (Roque-Barreira *et al.*, 1986). Bound IgA was eluted from the affinity column with 0·8 M galactose solution. The eluate was dialysed against phosphate-buffered saline (PBS, pH 7·4). Purity of the IgA preparation was determined by double

immunodiffusion and immunoelectrophoresis using specific goat anti-human IgM and IgG antisera (Meloy, Springfield, VA).

Preparation of IgA oligomers

A solution of purified IgA (2 mg/ml) in PBS was cooled in ice. A 20× molar excess (0·3 mM) solution of *N*-succinimidyl 3(2-pyridyldithio) propionate (SPDP) (Pierce Chemicals, Rockford, IL) in dimethylformamide was likewise cooled in ice. This solution was added dropwise to the IgA solution with gentle mixing. The mixed solution was kept at room temperature for 30 min with occasional stirring. The reaction mixture was then divided into two equal volumes. One-half of the solution was passed over Sephadex G-25 (1 × 10 cm) pre-equilibrated with PBS to remove unreacted SPDP and yield a 2-pyridyl disulphide solution. The other half of the reaction mixture was acidified with 0·1 M sodium acetate buffer (pH 4·5), containing 0·1 M NaCl. This solution was reduced with excess dithiothreitol (Calbiochem, La Jolla, CA) at a final concentration of 50 mM, and passed through Sephadex G-25 (1 × 10 cm) column, to obtain thiol modified IgA. The 2-pyridyl- and thiol-modified IgA solutions were conjugated by mixing at room temperature for 30 min followed by dialysis against borate-buffered saline (BBS). Chemically cross-linked IgA oligomers (X-IgA) were separated from the bulk of non-cross-linked IgA by passage over a column of Sephacryl S300 (2·6 × 95 cm) equilibrated with BBS. Effluent fractions containing predominantly large-sized X-IgA, as judged by gradient gel electrophoresis (Rifai & Mannik, 1983) were collected and concentrated under negative pressure in a dialysis colloidon sac immersed in BBS.

In order to maximize the selective recovery of large-sized X-IgA, 7% (w/v) polyethylene glycol (PEG) in BBS was added to the cross-linked IgA preparation (Imai *et al.*, 1987). The precipitated X-IgA was washed twice with 3·5% PEG, resolubilized with nine parts distilled water and the pH adjusted to 8·0 with one part 10X BBS.

Isolation of IgA immune complexes

Naturally occurring IgA-IC were isolated from the plasma of a 45-year-old patient treated with plasmaphoresis for idiopathic thrombocytopenia purpura and plasma cell dyscrasia. The plasma contained a high titre of IgA with rheumatoid factor activity. The IgA-IC were precipitated with ammonium sulphate and subjected to affinity purification as described above. Immunoelectrophoresis revealed that the IgA-IC consisted of IgA (κ) and IgG (λ). The purified IgA-IC were passed through a column of Sephacryl S300 (2·6 × 95 cm) equilibrated with BBS. Effluent fractions containing large-sized IgA were collected and concentrated as described above.

In some experiments, an equal volume of 7% PEG was added to the affinity purified IgA-IC to maximize the recovery of large-sized complexes. The PEG precipitate was washed twice with 3·5% PEG and resolubilized as described above.

Complement assays

Two different assays were used to quantitate C3 activation. C3 conversion was quantitated by the loss of the B antigen of C3 (West *et al.*, 1966; Marder *et al.*, 1983). Fifty microlitres of test reagent in 0·01 M PBS was incubated at 37°C with 450 μl of undiluted fresh normal human serum. The B antigen was measured by radial immunodiffusion in 1% agarose containing goat antiserum made specific for the B determinant by absorption with aged human serum. This antiserum was kindly provided by Ms J. Forristal and Dr C. D. West, Cincinnati Children's Hospital and Research foundation, OH. C3 conversion was expressed as a percent of the initial B antigen concentration. The test reagents, dissolved in PBS, examined in this assay were I-IgA (1 mg/ml), X-IgG (1 mg/ml), heat-aggregated human IgG (1 mg/ml), C3 nephritic factor (C3 NeF), and control buffer alone.

Heat-aggregated human gamma globulin (HAG) was prepared as described (Magilavy, Rifai & Plotz, 1981). Cross-linked IgG oligomers were prepared with SPDP as described above. The C3NeF was isolated from the serum of a terminally ill patient with marked hypocomplementaemia, respiratory failure and presumed septicaemia. The purification and characterization of this preparation was described (Waldo *et al.*, 1985). One part of the C3NeF was added to nine parts of serum for the activation experiments.

C3 activation was also assessed by generation of iC3b after a 30 min incubation of 100 μl of normal human serum with 100 μl of test reagent in BBS. An enzyme immunoassay kit (Cryotech, San Diego, CA) was used for quantitation of iC3b formed in serum. The monoclonal antibody utilized in this assay recognized only iC3b and not native C3, C3c or C3d (Kolb *et al.*, 1985; Tamerius, Pangburn & Mullar-Eberhand, 1985).

Factor B cleavage was measured semiquantitatively by radial immunodiffusion using an antiserum containing antibodies to Ba and native B/Bb (kindly provided by Drs J. Forristal and C. D. West). When factor B is cleaved, double-concentric precipitin rings are formed with the Ba as an outer ring and B/Bb as the inner ring (Marder *et al.*, 1983). Activation was measured as percent expansion in the area of the inner precipitin ring produced by untreated serum. It was subsequently subtracted from the larger ring area produced by the serum after it had reacted with a complement activator.

Animal experiments

Female 6-week-old BALB/c AnCrlBR mice (Charles River Breeding Laboratories, Wilmington, MA) were used in these studies. The experimental groups (3 mice/group) used for studies of glomerular localization of IgA and C3 were treated with: (a) soluble large-sized X-IgA; (b) soluble large-sized IgA-IC; (c) HAG; (d) X-IgG. Each mouse received a total of 2·8–3·0 mg of oligomers in four equal doses over a period of 2 h; the mice were killed 4 h after the last injection.

A portion of the mouse renal cortex was snap-frozen in embedding medium, and cut with a cryostat into 4 μm sections. The sections were air-dried, fixed in acetone for 10 min at room temperature and washed with PBS prior to incubation with fluorescein-labelled antiserum. Antisera, diluted 1:20, used were goat anti-human IgA and goat anti-mouse C3 (Cappel, Malvern, PA). Slides were incubated for 30 min at 37°C in a humid chamber washed with PBS and mounted with PBS glycerol.

RESULTS

Description of IgA immune complexes

Approximately 38% of the total IgA purified from the patient's serum consisted of large- ($> 10^6$ m.w.) and intermediate-sized ($0{\cdot}4\text{-}1 \times 10^6$ m.w.) complexes. These complexes were separated from dimeric and monomeric IgA by gel filtration on 2·6 × 95·

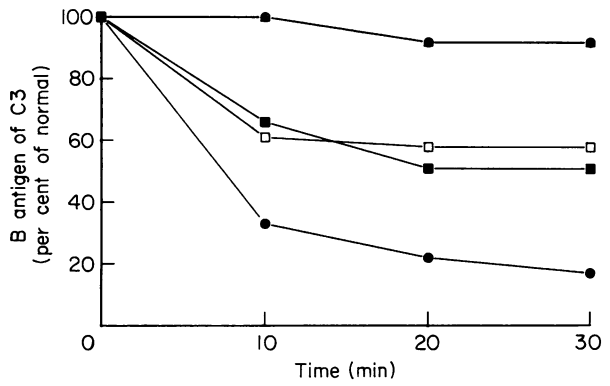

Fig. 1. Effect of 200 μg large-sized X-IgA oligomers (▲), 50 μg of X-IgG (■), 50 μg of heat-aggregated human gamma globulin (□), and 50 μl of C3 nephritic factor (●) on C3 conversion in normal human serum (○). C3 conversion is quantitated by the loss of the B antigen of C3 at various intervals.

cm columns of Sepharcryl S-300. Fractions containing large-sized complexes eluted between 39 and 46% of the total column volume were pooled and used in the subsequent experiments. Pooled fractions containing either dIgA and mIgA were used as control. The purified IgA-IC containined only IgA and IgG as determined by immunoelectrophoresis and double immunodiffusion (data not shown).

Preliminary experiments had indicated that the heterobifunctional cross-linking reagent SPDP was most effective when mixed with IgA at 20:1 molar ratio. Approximately 60% of the total IgA was polymerized into large- and intermediate-sized oligomers. The covalent cross-linking of these oligomers was verified by sodium dodecyl sulphate gradient polyacrylamide gel electrophoresis. The gel filtration pattern of the X-IgA was similar to that of the naturally occurring IgA-IC. Eluates containing either large-sized X-IgA, dIgA, or mIgA were pooled and concentrated by negative pressure.

In vitro analysis of C3 and factor B activation

Monomer, dimer, intermediate- and large-sized IgA-IC or X-IgA preparations were tested for their ability to cleave C3. Under the conditions used in these experiments, 42–49% decrease of the initial C3 in 0·45 ml of serum was induced in 30 min by 50 μg of the standard heat aggregated normal human IgG or X-IgG (Fig. 1). Another control, C3NeF (50 μl), resulted in 83% decrease of the initial C3. In contrast, 200 μg of all the IgA preparations failed to alter significantly the initial C3.

The ability of IgA-IC to activate C3 was also evaluated by quantitative measurement of the neoantigen iC3b in treated normal serum. In these experiments large-sized IgA-IC and X-IgA were isolated by precipitation with 3·5% PEG. Unlike the naturally occurring IgA-IC, the X-IgA precipitates failed to resolubilize and were used as such. As illustrated in Fig. 2, no significant difference in the amount of iC3b was generated in normal serum treated with 1 mg of either unreacted IgA, soluble IgA-IC, or insoluble X-IgA. By comparison 1 mg of soluble heat-aggregated human gamma globulin was very effective in generating iC3b. These results suggest that soluble or insoluble IgA-IC are incapable of activating C3.

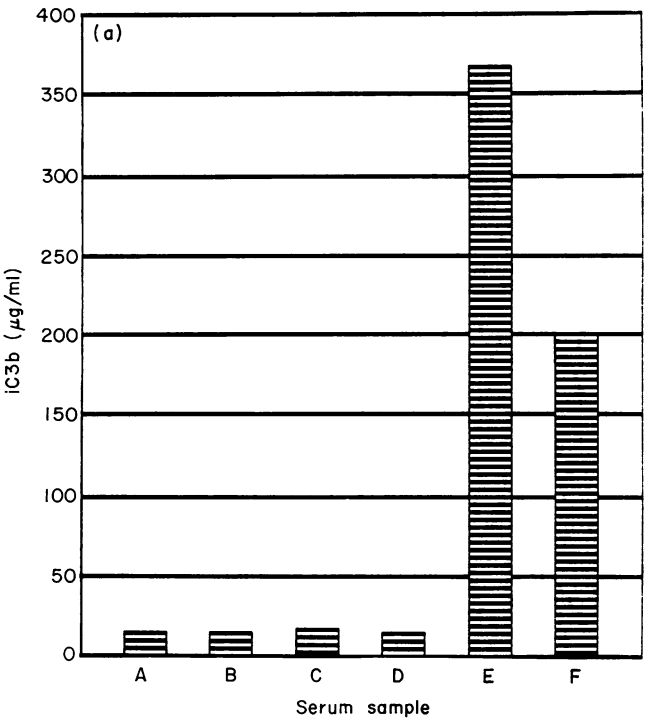

Fig. 2. The concentration of iC3b (μg/ml) in normal serum specimens treated with: (A) buffer; (B) 1 mg/ml of purified IgA; (C) 1 mg/ml of insoluble X-IgA; (D) 1 mg/ml soluble IgA-IC; (E) 1 mg/ml soluble HAG; (F) 20 mg/ml inulin.

The IgA-IC and X-IgA were also tested semiquantitatively, by single radial immunodiffusion, for ability to induce in normal human serum the generation of Ba and Bb breakdown products. Large-sized IgA-IC or X-IgA (200 μg) failed to generate Ba in normal serum. By comparison, 50 μg of HAG or 50 μl of C3NeF resulted in greater than 50% increase in the expansion of the precipitin ring. These results indicate there was no consumption of Factor B by soluble IgA-IC or X-IgA.

In-vivo analysis of C3 activation

The possibility that IgA-IC or X-IgA acquire the ability to activate C3 after glomerular deposition was also examined. A total of 3 mg of large-sized soluble IgA-IC, X-IgA, HAG or X-IgG, was administered intravenously to each mouse in the experimental groups.

Immunofluorescent examination of the renal tissues, obtained 6 h after the initial injection, demonstrated the presence of glomerular IgA deposits without a concomitant deposition of C3 (Fig. 3). In contrast, concomitant glomerular deposits of IgG and C3 were detactable in mice treated with HAG or X-IgG (data not shown).

DISCUSSION

Naturally occurring IgA-IC and chemically cross-linked oligomers of human IgA were used to investigate activation of the complement system *in vitro* and *in vivo*. Both IgA-IC and X-IgA oligomers were unable to induce the cleavage of C3 or factor B in normal human serum. Passive infusion of both types of complexes into mice resulted in glomerular deposition without concomitant induction of C3 deposits.

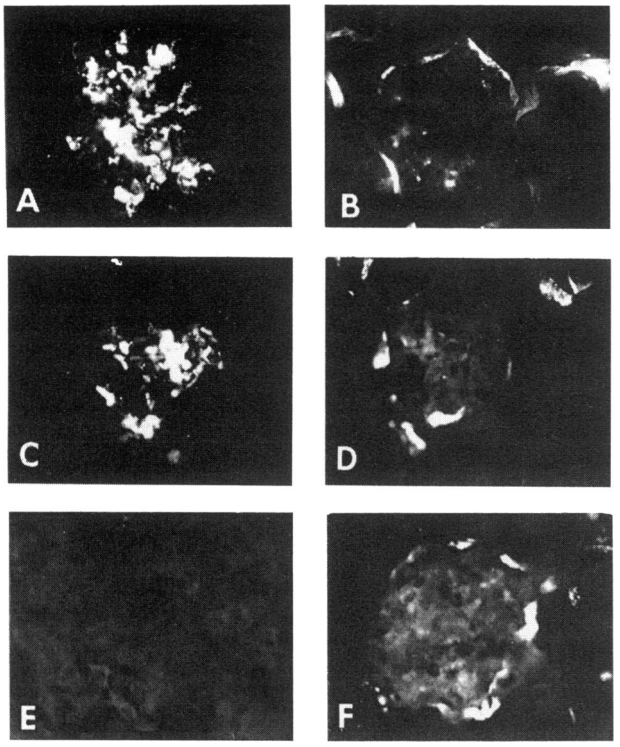

Fig. 3. Representative immunofluorescent staining of IgA deposits in glomeruli of mice that received either 2·8 mg/IgA-IC (A) or 3·0 mg X-IgA (C). Lack of C3 deposition is demonstrated in the IgA-IC (B) or X-IgA (D) treated mice. Normal mouse glomerulus stained with anti-IgA (E) and anti-C3 (F). Note: goat anti-mouse C3 reacts non-specifically with Bowman's capsule and tubular membranes.

Activation of C3 plays a major role in both the classical and the alternative pathways. We utilized two different, but complementary, assays for the detection of C3 activation. In the first assay, the loss of the B-antigenic determinant of C3 was evaluated. This antigenic determinant is present only on the native C3 molecule and cannot be detected by immunological methods on any breakdown products (West *et al.*, 1966). In the second assay, a highly sensitive enzyme-linked immunoasay was used to quantitate the inactivated C3b (iC3b) generated by proteolytic cleavage of the α-chain of C3b (Tamerius, Pangburn & Muller-Abehard, 1985). In this method, a unique monoclonal antibody specific for a neoantigen present on iC3b was used to quantitate C3 activation. Both assays were consistent in demonstrating that IgA-IC and soluble or insoluble IgA oligomers were unable to activate C3. Inability to activate the alternative pathways was further demonstrated by the failure of different IgA preparations to result in cleavage of factor B in the treated serum.

These results appear to contradict a recent report on activation of the alternative pathway of complement by human serum IgA (Hiemstra *et al.*, 1987). The difference in the findings warrants the consideration of several points. First, those studies depended on a modified haemolytic assay of sheep red blood cells coated with murine monoclonal (IgG1) anti-human IgA. It is well documented that mouse IgG1 aggregates are capable of activating the alternative pathway (Ey, Prowse & Jenkin, 1979; Klaus *et al.*, 1979). The potential participation of such aggregates in activation and subsequent haemolysis of the indicator cells could not be ruled out. Second, large-sized heat-aggregated IgA and other chemically cross-linked IgA oligomers either failed or were inefficient in the induction of haemolysis. This finding, although not emphasized, corroborates our report. Third and most important, the SPDP-treated IgA monomers were as effective as the oligomers in inducing haemolytic activity. This would suggest that the observed haemolytic activity may have been due to a thiol group on SPDP-treated IgA that reacted with either the membrane or the IgG1 on the surface of the indicator cells.

Clinically, it is unknown how C3 and other complement components localize in the glomeruli of patients with IgA nephropathy. It is noteworthy, however, that two comprehensive clinical studies showed that only 55% of the patients with exclusively IgA immune deposits had concomitant C3 glomerular deposits (Shirai *et al.*, 1978; Okada 1985). In contrast, 85–90% of the patients had C3 deposits when IgG and/or IgM was co-deposited with IgA. Thus, other classes of immunoglobulin may be responsible for C3 deposition. In addition, the potential role of the antigen in mediating complement activation has been recently documented in an experimental model of IgA nephropathy (Rifai, Chen & Imai, 1987). Lack of C3 activation by the glomerular deposits of human IgA-IC or X-IgA (Fig. 3), are in agreement with those observations. Collectively, the *in vitro* and *in vivo* presented data support our suggestion that human IgA in an aggregate or in a complex-form lacks the ability to mediate complement activation.

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